How cells process information: Quantification of spatiotemporal signaling dynamics

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Received 29 March 2012; Accepted 23 April 2012

DOI: 10.1002/pro.2089

Published online 9 May 2012 proteinscience.org

Abstract: Arguably, one of the foremost distinctions between life and non-living matter is the ability to sense environmental changes and respond appropriately—an ability that is invested in every living cell. Within a single cell, this function is largely carried out by networks of signaling molecules. However, the details of how signaling networks help cells make complicated decisions are still not clear. For instance, how do cells read graded, analog stress signals but convert them into digital live-or-die responses? The answer to such questions may originate from the fact that signaling molecules are not static but dynamic entities, changing in numbers and activity over time and space. In the past two decades, researchers have been able to experimentally monitor signaling dynamics and use mathematical techniques to quantify and abstract general principles of how cells process information. In this review, the authors first introduce and discuss various experimental and computational methodologies that have been used to study signaling dynamics. The authors then discuss the different types of temporal dynamics such as oscillations and bistability that can be exhibited by signaling systems and highlight studies that have investigated such dynamics in physiological settings. Finally, the authors illustrate the role of spatial compartmentalization in regulating cellular responses with examples of second-messenger signaling in cardiac myocytes.

Keywords: signaling molecules; biosensors; fluorescence; computational modeling; feedback; quantitative biology

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Grant sponsor: NIH Director's Pioneer Award; Grant number: DP1 OD006419; Grant sponsor: NIH; Grant number: R01 DK073368 (to Jin Zhang); Grant sponsor: American Heart Association; Grant number: 11PRE7660055 (to Ambhighainath Ganesan).

Introduction

Cells use signaling cascades to make critical and complicated decisions in response to external stimuli in multiple contexts—apoptosis, metabolism, and migration, to name a few. However, the process of cellular signaling in its most elemental form comprises nothing but a set of chemical reactions—perhaps, an old phenomenological remnant from the

days of the proto-cells, which were likely little more than membranous bags of reacting molecules.¹ This mismatch between the complexity of functional responses and the apparent simplicity of the underlying molecular mechanisms has been bridged by living forms during the course of evolution primarily in two methods: (a) by expanding the repertoire of signaling molecules (e.g., various post-translationally modified forms and splice variants) (b) by exploiting temporal and spatial variations to diversify the portfolio of responses using the same set of signaling molecules.

Temporal changes in concentrations or activities of effector molecules in a signaling cascade occur commonly in response to external stimuli. Depending on the architecture of the signaling network, these temporal changes may be precisely controlled and coordinated to exhibit complex dynamics such as adaptation or oscillations, for instance. Oscillations, in fact, commonly occur in many organisms ranging from unicellular bacteria to mammalian cells. Temporal oscillations are thought to regulate metabolism,^{2,3} cell cycle,⁴ circadian rhythms,^{5,6} and rhythmic beating of the heart⁷ among many other physiological processes. Alternately, signaling cascades could translate information about external stimuli to spatial dynamics of signaling molecules. For instance, in fibroblasts, inhomogeneous spatial distribution of signaling activities associated with cytoskeletal reorganization, such as Rac and Cdc42 GTPase activities, influence cell migration.⁸ Understanding the biophysical bases of spatiotemporal dynamics of signaling is therefore important in appreciating how cells process information from external cues to regulate cellular processes of biomedical importance. Researchers have been able to uncover mechanisms underlying signaling dynamics using a vast array of experimental tools and modeling approaches some of which the authors briefly outline in the following sections.

Experimental tools for monitoring signaling dynamics

Technological advances in microscopy and biosensor development over the past two decades have greatly facilitated the observation of spatiotemporal dynamics of signaling molecules in their native environment. Small ion second messengers such as calcium can be tracked using dye indicators such as Fura-2, some of which were developed more than 20 years ago and are still commonly used. On the other hand, monitoring other signaling molecules such as kinases, phosphatases, and cytoskeletal proteins often requires cell lysis or fixation followed by immunoblotting and immunocytochemistry, where real-time information is often lost.^{9–13} The advent of fluorescent protein (FP) technology combined with advancements in microscopy has largely addressed these

issues by making it possible to observe signaling dynamics in live-cell context with millisecond temporal resolution and tens of nanometer spatial resolution. An important frontier of this technology has been the development of suites of genetically encoded fluorescent biosensors for monitoring spatiotemporal dynamics of signaling molecules.

The simplest generic fluorescent biosensor design consists of a FP fused to the protein of interest or a protein-domain. One such popular biosensor used to detect phosphoinositide dynamics comprises the enhanced green FP (EGFP) fused to the Pleckstrin homology (PH) domain of Akt [Fig. 1(A)].¹⁴ This biosensor exploits the ability of the PH domain to bind to 3' phosphoinositides at the plasma membrane. Accordingly, increased PIP₃ synthesis is converted into an optical readout in the form of increased fluorescence intensity at the membrane.

Another popular class of single FP-biosensors exploits changes in fluorescence of individual fluorophores to indicate physiological events. The calcium indicator, GCaMP, comprises a circularly permuted GFP fused to calcium-binding calmodulin (CaM) and CaM-binding M13 peptides [Fig. 1(B)].¹⁵ In the calcium-unbound state, the chromophore has direct access to the solvent and so the chromophore exists in a non-fluorescent protonated form. Binding of calcium, however, induces a conformational change leading to blocking of solvent access to the chromophore and thus “switch on” the fluorescence.¹⁶ The latest published variant, GCaMP3, boasts a dynamic range of as high as about 300% and can be used to monitor calcium changes *in vivo*.¹⁷ Recently, Campbell and colleagues have expanded the suite of single FP calcium biosensors using FPs of different hues ranging from blue to red.¹⁸ These sensors hold big promise for multicolor imaging of different FP-based biosensors. The red-shifted calcium sensors are also optimal for simultaneous use with blue-light activatable optogenetic tools such as Channelrhodopsin.^{19,20} Most of these single FP-biosensors however are intensity-based and are susceptible to motion artifacts, variations in intensity of excitation light and other optical parameters, and can therefore be complemented by ratiometric indicators.

One way of developing ratiometric indicators is to exploit Förster resonance energy transfer (FRET) between a fluorophore pair. A major class of FRET-based biosensors makes use of conformational changes induced upon ligand binding or post-translational modifications (See Refs. 21–23 for a comprehensive review on FRET-based biosensors). For instance, the generic design for kinase activity reporters (KARs)^{24–27} consists of a substrate peptide and a phospho-amino acid binding domain “sandwiched” between two FPs. Phosphorylation of the substrate by the kinase of interest and the subsequent binding of the phosphorylated substrate to the

Figure 1. Fluorescence-based biosensors. **(A)** A PIP₃ biosensor contains the PH domain of Akt tagged to EGFP. An increase in the synthesis of PIP₃ leads to recruitment of the biosensor to the membrane and hence increased fluorescence in the membrane. **(B)** The calcium biosensor, GCaMP contains a circularly permuted EGFP (cpEGFP) sandwiched by a Calmodulin (CaM) peptide and the CaM-binding M13 fragment of myosin light chain kinase. Increase in calcium concentration leads to binding of calcium-CaM to M13 thereby filling up a cavity in the cpEGFP and hence increasing fluorescence intensity. **(C)** A generic genetically encoded FRET-based kinase activity biosensor contains a target substrate peptide and phospho-amino acid binding peptide flanked by a cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Increase in kinase activity leads to phosphorylation of the target substrate domain. Subsequent conformational change upon binding of kinase substrate domain to the phospho-binding domain brings the two fluorescent proteins together resulting in increased FRET.

phospho-amino acid binding domain results in a change in the distance and/or relative spatial orientation of the two FPs and hence a change in FRET between them [Fig. 1(C)]. The KARs have become a valuable tool in elucidating mechanisms of kinase-mediated signaling regulation.

Protein kinase A (PKA) is a prototypical kinase involved in multiple signaling pathways and regulates numerous cellular processes ranging from fibroblast cell migration to cardiomyocyte contractility. PKA activity dynamics has been observed in real-time using the A kinase activity reporter (AKAR), which has helped then authors uncover new insights into kinase regulation in physiological processes. A plasma membrane targeted AKAR biosensor has been used to observe PKA activity in migrating fibroblasts enabling researchers to discover that PKA is activated at the leading edge of

migrating cells.²⁸ Subsequently, activated PKA phosphorylates RhoA, regulating the cycling of RhoA activity at the leading edge and thus sets the frequency of protrusion–retraction cycles.²⁹ Extending this approach to other kinases has also made it possible to characterize the interplay between different kinase signaling modules to interpret external cues. Recently, AKAR and the ERK activity reporter (EKAR) have been used together to delineate PKA and ERK crosstalk in PC12 cells in response to growth factor stimulation.³⁰

Computational Modeling-Aided Approaches to Discern Signaling Dynamics

Apart from a host of experimental tools detailed above, computational modeling is increasingly becoming an indispensable resource for researchers

Figure 2. Modeling-driven dissection of signaling networks. Known non-linear connections (denoted by dark lines) in a network comprising three components *A*, *B*, and *C* are represented by a mathematical model. In this network, *A* activates *B*, *B* activates *C*, and *C* inhibits *A* in a negative feedback manner. Simulations of the model can then be matched with the experimental data. Any discrepancies between experiments and model can indicate the need for further improvements to the model, typically involving a revision of parameter values. Simulations can also reveal the presence of new connections (shown in dotted lines) leading to the formulation of a more complete network which can better explain experimental results.

studying signaling dynamics. Quantitative models are especially helpful in the case of large signaling systems, in which individual components may not necessarily interact in ways that can be fully predicted based on the properties of these components studied separately, but may instead acquire emergent properties endowed by complex intermolecular interactions. Such systems-level properties make it difficult to predict system responses intuitively (Fig. 2). In particular, the emergent properties can engender complex dynamical responses, including oscillations or multistability,^{31,32} requiring modeling approaches for in depth understanding and analysis. Further, models can be powerful tools in generating and testing hypotheses quickly and inexpensively, leading to rational design of subsequent experiments. Models can also be powerful in generating quantitative paradigms integrating diverse data into a single framework and helping abstract the underlying evolutionary “design” principles of signaling networks,^{33,34} which may be subsequently used in reverse engineering of novel signaling networks.^{35,36}

Biological signaling reactions, like their chemical counterparts, are subject to the laws of mass action and can therefore be mathematically represented in the form of ordinary differential equations

(ODEs). An ODE, describing the rate of change of concentration of a molecular species, captures the balance (or imbalance) between the synthesis and degradation of a particular species, or its activation/inactivation through chemical modification. A system of ODEs can therefore theoretically predict the changes in the concentrations of the modeled species over various periods of time. Although signaling networks are complex, the first step toward modeling a new system is to make appropriate assumptions and build a simple ODE model consisting of two or three states based on the kinetic information available. Such small-scale models often give remarkable insight into the major functional responses of the system. A regularly used mathematical tool, in such cases, is the phase portrait, which describes how the concentrations of different species would vary coordinately with time under different initial conditions. Phase portraits can be immensely helpful in not just determining the steady states of the system but also in predicting if the system is capable of exhibiting dynamical instabilities such as oscillations or multistability.³⁷

Predictions from simple models can drive further experimentation. As more experimental results and more detailed kinetic information become

Cytosol: $\frac{dA_c}{dt} = (k_{fc} - k_{rc}A_c) + (k_{nc}A_n - k_{cn}A_c)$

Nucleus: $\frac{dA_n}{dt} = (k_{fn} - k_{rn}A_n) + (k_{cn}A_c - k_{nc}A_n)$

Figure 3. A representative compartmentalized model of spatial and temporal dynamics of the activity of a species, A. The cell is divided into two homogenous compartments: the cytosol and the nucleus. A is generated and degraded in the cytosol at rates k_{fc} and k_{rc} , respectively, and is generated and degraded in the nucleus at rates k_{fn} and k_{rn} , respectively. Further, A is assumed to shuttle between the two compartments at effective rates of k_{cn} and k_{nc} . Separate ODEs for each compartment are written by summing up the reaction and diffusion rates.

available, it becomes possible to develop more sophisticated ODE models with 10 or more states, which can adequately capture the complex and finer details of the system. However, the large number of parameters in such large-scale models can lead to the problem of over-fitting the experimental data. This problem can be addressed by undertaking parameter sensitivity analysis.³⁸ In this analysis, the model is simulated with variation in one or more parameter values and features of the system response are compared with those in unaltered, nominal model to develop, and evaluate appropriate sensitivity metrics. Such metrics can indicate which parameter sets can change the model response dramatically and variation of which are of considerably less consequence. Further experiments may then be required to get more accurate kinetic information for the “sensitive” parameters.

ODE models have been widely used to describe temporal dynamics of signaling pathways. However, signaling molecules also exhibit spatial dynamics which can be important functionally. Arguably, the easiest approach to model spatial and temporal dynamics is to use ODE-based compartmentalized-models. In such models, the cell is assumed to consist of different discrete compartments such as the plasma membrane, cytosol, and nucleus. Temporal dynamics in each compartment is described by a separate set of ODEs, and a different set of ODEs is used to describe the flux of signaling molecules between compartments (Fig. 3). However, compart-

mentalized-models may not be adequate to model more intricate spatial dynamics inside cells such as phosphorylation gradients^{39,40} or calcium microdomains.^{41,42} Partial differential equations (PDEs) are therefore used instead of ODEs in such cases. PDEs are multivariable equations that mathematically describe the change in concentrations of different species across both space and time. PDE models have been used to describe complicated spatial dynamics in multiple systems ranging from patterning of developing *Drosophila* embryos⁴³ to ameboid fruiting body formation.⁴⁴

While most signaling models are deterministic in nature, the role of noise in decision making by signaling circuits is increasingly being appreciated. Stochasticity is particularly relevant in situations where small numbers of molecules are involved in a reaction. Stochasticity in signaling systems can be most simply modeled by the addition of one or more Gaussian terms, representing noise, to an existing ODE resulting in stochastic differential equations. Alternatively, small-scale systems can be modeled using the stochastic simulation algorithm (SSA), which essentially describes the random walk behavior of the molecules in a reaction.⁴⁵ However, since the SSA simulates every event in a set of reactions, it can become computationally expensive for large systems. Nonetheless, improvements have been made to the SSA to overcome this issue, albeit with some loss in the exactness of the algorithm.⁴⁶

Temporal signaling dynamics

Intracellular responses to an external stimulus often involve temporal changes in concentrations or activities of specific signaling molecules. Cells have therefore evolved to use variations in temporal dynamics as a means of inferring and reacting to external stimuli. In this article, the authors focus on a few examples of temporal dynamics.

Cellular signaling cascades are complex systems embedded with non-linearities in the form of feedback loops. The presence of negative feedback loops can produce adaptative responses to a constant stimulus [Fig. 4(A)]. Adaptative responses, such as those in bacterial chemotaxis, can be especially helpful in responding to repeated stimuli. Given the right conditions, such as sufficient time-delay, a circuit with negative feedback loop can also produce more dynamically complex behavior such as oscillations [Fig. 4(B,C)]. Oscillations have been recognized as a rich means of communication within the cells. Cells have evolved to use the same oscillating signal to convey different outcomes through simple changes in the parameters of oscillations such as frequency and amplitude.^{47,48} Calcium oscillations, in particular, occur in different cell types, both excitable and non-excitable. Neuronal calcium spiking has been linked to, among many other things, memory

Figure 4. Temporal dynamics of signaling circuits with negative feedback loops in a representative system comprising two molecules, *X* and *Y*. **(A)** The presence of a negative feedback loop alone can produce a transient adaptative output signal in response to a “step-input” signal. **(B)** Negative feedback loop coupled with time-delay [represented explicitly as $Y(t) = X(t - \tau)$] produces a damping oscillation in response to a “step-input” signal. **(C)** Negative feedback with time-delay (represented by the presence of an extra species, *Z* in the circuit) coupled with additional positive feedback loops results in sustained oscillations in response to a “step-input” signal. Positive feedback loops are denoted in green. Negative feedback loops are denoted in red.

formation through the actions of calmodulin kinase II and other calcium-sensitive proteins.^{49,50} In pancreatic β -cells, calcium oscillations are believed to determine the rhythmic secretion of insulin, which is critical for glucose homeostasis.^{51,52} Calcium oscillations are also known to regulate the contractility of cardiac myocytes through calcium-sensitive contractile proteins.⁵³ The authors and others have used experimental monitoring of temporal dynamics of signaling activities in conjunction with computational models to address fundamental questions pertaining to intracellular communication.

Recently, the authors demonstrated in pancreatic β -cells that PKA activity oscillates in a calcium-dependent manner upon membrane-depolarization.⁵⁴ Further, PKA activity also appears to be necessary for calcium oscillations indicating the presence of a feedback loop from PKA to calcium. Based on these

experimental observations, the authors built a mechanistic ODE model. Model predictions indicated that increasing the strength of PKA feedback to calcium could result in modulation of frequency of calcium oscillations, which was subsequently verified through experiments. As such, combined experimental and modeling results revealed the existence of an oscillatory circuit consisting of calcium, cAMP, and PKA—where PKA serves a frequency modulator. The model also helped generate the hypothesis that PKA activity oscillations might result in spatially selective phosphorylation of target substrates, as was confirmed through experiments. The authors observed that conditions leading to low frequency oscillations restrict PKA activity to local signaling domains and prevent it from propagating into the nucleus, whereas high frequency oscillations result in global PKA signaling. Thus the oscillations allow the system to achieve signaling specificity and diversity.

Combined experimental–computational approaches have also been used to understand the mechanisms of oscillations of other molecules such as the transcription factor nuclear factor kappa B (NF- κ B), which plays critical roles in mammalian inflammatory immune responses. NF- κ B activity is primarily subject to regulation by the Inhibitor of NF- κ B (I κ B) protein which binds to NF- κ B and sequesters it from the nucleus. Various stimuli can result in the phosphorylation and degradation of I κ B which enables the free NF- κ B to translocate to the nucleus and activate the transcription of its target genes. A joint experimental–modeling study by Hoffman et al.⁵⁵ demonstrated that the presence of different isoforms of the I κ B protein could modulate the strength of a negative feedback loop involving NF- κ B and I κ B, resulting in varied temporal dynamics of NF- κ B activity oscillation. These oscillations can play important roles in the cellular decision making processes. For instance, recently single-cell imaging coupled with stochastic mathematical modeling revealed that cells can decode oscillation properties such as peak intensity and peak width to modulate NF- κ B activation.⁵⁶

Whereas negative feedback loops result in oscillatory dynamics, the presence of positive feedback loops can bring about drastically different dynamics. Under certain conditions (Fig. 5), a signaling circuit with positive feedback loop can result in bistability (in general more, multistability), in which case the system has the ability to reside in one of two given steady states—“on” or “off.” In other words, cells can theoretically exist only in either of these two states with other intermediate states being “forbidden”; in reality this manifests as a bimodal distribution of steady state responses among a population of cells. The mitogen-activated protein kinase (MAPK) signaling cascades contain numerous feedback loops

Figure 5. Bistability in a circuit with a positive feedback. In a circuit with positive feedback loops, multiple steady state responses are possible. The simplest case with two steady states is referred to as a bistable system. In this schematic, the system can exist in either of two steady states, the “ON” state or the “OFF” state, for a particular range of values of a certain kinetic parameter, k (say). A hallmark of bistable systems is the phenomenon of hysteresis, in which, the system response depends not only on current conditions but also the path taken to reach the current conditions. In this case, the system transitions from the “OFF” state to the “ON” state at the parameter value k_1 , as k is increased. However, the system transitions from the “ON” state to the “OFF” state at the parameter value, k_2 different from k_1 , as k is decreased.

facilitating the occurrence of bistability. The c-Jun N-terminal kinase (JNK), belonging to the MAPK family, has been shown to exhibit bistability in *Xenopus* oocytes.⁵⁷ Bagowski and Ferrell observed that sorbitol-stimulated JNK activity in individual oocytes was bimodal in nature, indicating the presence of bistability. Further, they observed that JNK activity exhibited hysteresis, a typical hallmark of bistable systems, wherein the threshold for pushing the system to an “on” state from an “off” state is significantly different from the threshold for deactivating the system from an “on” state to an “off” state. Bistability in JNK signaling may also be present in mammals. Recently, our group has demonstrated that JNK shows an “all-or-none” behavior in mammalian cells in response to ribotoxic stress.⁵⁸ As in the case of JNK, bistability is also thought to occur in signaling cascades involving other MAPKs like ERK and p38 in mammals.^{59–61}

Spatial signaling dynamics

Simplistic models quite often assume cells to be homogeneous “bags” of molecules. While this first order of approximation may be sufficient to explain many temporal aspects of signaling, a cell, in reality, is made up of spatially restricted compartments that perform pre-defined sets of functions. Taking a simplistic view, the nucleus serves as the genetic memory bank, the mitochondria act as “power-houses” while the endoplasmic reticulum is involved in translation and folding of newly synthesized pro-

teins. Yet these organelles share the same “channels of communication” with the external world, namely, the cytosol and the plasma membrane. To achieve spatially distinct signals, the cell uses different mechanisms ranging from use of signaling molecules and pathways that are unique to each organelle to modulation of the concentrations of different pools of spatially universal signaling molecules such as calcium. Precise spatial dynamics of signaling is achieved by a combination of physical processes (diffusion), chemical factors (balance between the rates of synthesis and degradation of the signaling molecules), and structural factors (cellular structures, and scaffold or anchor proteins in the context of protein complexes) to sequester molecules in defined locations.

Calcium microdomains such as calcium “sparks” have been extensively studied in cardiomyocytes in the context of the excitation–contraction coupling (ECC) process. Calcium “sparks” arise from the spontaneous opening of a cluster of Ryanodine receptors (RyRs) resulting in local increases in calcium concentration.⁶² Apart from spontaneous generation, calcium “sparks” can also be evoked in cardiomyocytes during ECC when calcium influx across voltage-gated calcium channels on the plasma membrane triggers the opening of calcium-sensitive RyRs on the sarcoplasmic reticulum (SR). Calcium “sparks” are now widely recognized to be the unitary events underlying the ECC process. Indeed, the total calcium release flux through the SR during ECC is thought to be largely mediated by evoked sparks.⁶³ Consequently, calcium “sparks” have been demonstrated to mediate regulation of cardiac contractility.^{64,65} Research on spatial aspects of calcium dynamics in living cardiomyocytes has led to a better understanding of the physiological roles of calcium “sparks” as well as pathological implications of their dysregulation. Imaging of calcium dynamics in myocytes obtained from failing human hearts revealed that the spatial and temporal properties of calcium “sparks” were significantly altered in comparison to “sparks” in myocytes from non-failing hearts indicating that the calcium release from the SR may be altered in failing hearts.⁶⁶ Spatial aspects of calcium “sparks” appear to be particularly important for proper cardiac function. In failing hearts, a calcium “spark” can trigger “sparks” from adjacent clusters of RyRs, setting up calcium waves, which can ultimately result in cardiac arrhythmias.^{67,68} Mathematical modeling has been used to verify that the experimentally observed diffusion constant of calcium in SR is sufficient to increase local SR calcium in sites adjacent to a “spark” site. Increase in local SR calcium coupled with an increase in cytosolic calcium has been suggested to trigger the opening of the adjacent inactive RyRs, resulting in calcium waves.⁶⁹ Although the initiation and propagation of

calcium sparks has been studied extensively, important questions pertaining the termination of calcium “sparks” still remain unanswered. Integrated experimental–computational approaches may help us better understand this phenomenon and its role in heart failure.

Another second-messenger molecule, cAMP, also exhibits intricate spatial dynamics influencing multiple biological functions including cardiomyocyte contractility. Increase in cAMP is observed in cardiomyocytes following stimulation of either prostaglandin or β -adrenergic receptors; yet the functional outcomes are markedly different in the two cases.^{70–72} Compartmentation of cAMP signaling has been proposed as a mechanism to explain how the same signaling molecule can control diverse processes in response to different stimuli. The use of fluorescent reporters of cAMP has led researchers to directly confirm the presence of cAMP compartmentation within single cells, in cardiac myocytes and other cell types.^{73–76} The physiological effects of cAMP signaling are largely mediated by the cAMP effector, PKA. Gradients in intracellular cAMP are therefore expected to result in non-uniform spatial PKA activity to effect diverse outcomes to different stimuli. In fact, PKA kinetics in cardiac myocytes is faster in the plasma membrane when compared with that in the cytosol membrane in response to β -adrenergic stimulation.⁴⁰ Saucerman et al. used a two compartment model to describe cAMP diffusion and the resulting PKA dynamics. Expectedly, cAMP diffusivity was found to influence PKA phosphorylation delay between plasma membrane and cytosol. To test the role of cAMP diffusivity on spatial PKA dynamics, a photo-activable cAMP analog was uncaged locally leading to a PKA phosphorylation gradient across the cytosol. Further the model helped identify potential signaling mechanisms for regulating spatial PKA activity dynamics. Both PKA-mediated buffering of cAMP and restricted cAMP diffusion mediated by PDEs help establish the phosphorylation gradients.

Conclusion

Signaling networks have been studied for well over six decades with a large focus on discerning connections between different components of signaling cascades, perhaps motivated by the availability of traditional genetic manipulation techniques. Recently, however, advances in microscopy and FP technology have enabled researchers to unearth more general principles of signaling pertaining to spatiotemporal dynamics. Studying signaling dynamics has helped us understand how cells process information; not as static entities but as constantly changing systems.

Cell cultures have been the preferred models for studying signaling dynamics; however, it is very likely that *in vitro* dynamics may be significantly

different from *in vivo* dynamics.⁷⁷ A more complete understanding of the role of signaling dynamics in animal physiology therefore requires the ability to monitor signaling molecules not in test tubes or cell culture dishes but in live animals. A subset of these tools such as the GCaMPs has already been used to probe signaling in live animals; however, an improved set of technologies and tools, for example, activity biosensors based on infrared FPs⁷⁸, and optical fiber-based deep tissue imaging,⁷⁹ are required to probe signaling molecules in different native contexts of live animals.

Our understanding of signaling dynamics can also improve with increased knowledge of the underlying architecture of signaling networks. Connections between various signaling entities are traditionally inferred using small molecule inhibitors and/or RNA interference to perturb activities of signaling molecules. However, most of these tools suffer from various limitations. Small molecule inhibitors can be non-specific and small interfering RNA (siRNA) molecules cannot be used to regulate target activity with precise temporal or spatial control. A more amenable approach would be to directly and specifically perturb the concentration or activity of a signaling molecule and then measure the response of its immediate downstream targets. Second-messenger molecules like calcium and cAMP have been perturbed using cell-permeant photolabile chelators.^{80,81} Rapamycin-inducible protein heterodimerizing systems^{82,83} and multiple photo-activable signaling molecules^{84–86} have now been demonstrated to provide acute target perturbation. Most of these tools are also genetically encodable making it easier to perturb signaling networks in particular cell types or even sub-cellular locations specifically. Further these tools are suited to be combined with fluorescent biosensors to interrogate signaling circuit connections and monitor the signaling responses at the same time.

Efforts to infer signaling network connections can also greatly benefit from the development of high-throughput technologies for simultaneously monitoring activities of multiple signaling molecules. A microfluidic platform dubbed ImStain carries out high-throughput immunostaining and has been used to measure signaling activities of up to four different molecules simultaneously in a single device.⁸⁷ Advancements to such devices enabling the measurement of multiple signaling activities in real-time can greatly aid large-scale dissection of signaling networks. Lastly, adaptation of theoretical tools from other disciplines may also help authors infer connections between signaling molecules precisely and quantitatively. Granger-causality, a commonly used tool in Economics to infer causative correlations between two entities based on time series data, has been put forward as a likely candidate tool for

inferring connections in signaling biology.⁸⁸ A confluence of such theoretical and experimental tools can therefore greatly accelerate studies aimed at understanding intracellular signaling dynamics and help us better understand how cells process information.

Acknowledgment

The authors thank Prof. Andre Levchenko for helpful comments on modeling methods.

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